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THE REGULATORY ROLE OF MICRORNA IN HUMAN ADIPOCYTES AND THEIR LINK TO INSULIN RESISTANCE

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The regulatory role of microRNA in human adipocytes and their link to insulin resistance

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

White adipose tissue (WAT) is a highly plastic organ which secretes a large number of peptide factors termed adipokines. Increased caloric intake increases WAT mass which results in marked alterations in the secretory pattern. This may be mediated via both transcriptional and post-transcriptional mechanisms. Obesity is associated with several complications including type 2 diabetes mellitus (T2DM), hypertension, cardiovascular disease and cancer. The overall aim of this thesis was to identify microRNAs (miRNA) in obese WAT and to determine if they regulate genes, in particular those encoding adipokines that are linked to obesity-associated insulin resistance.

In **Study I**, we investigated miRNA regulation of Chemokine (C-C Motif) Ligand 2 (CCL2) by extending and validating a recently identified transcriptional regulatory network in human adipocytes. The updated subnetwork predicted that miR-126-3p, miR-193b-3p and miR-92a-3p control CCL2 production through several transcription factors (TFs) such as v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), MYC-associated factor X (MAX), and specificity protein 1 (SP1). Selective down-regulation of ETS1, MAX, or SP1 in human adipocytes attenuated CCL2 production and a concomitant gene silencing of ETS1 and MAX resulted in an additive reduction in CCL2 production. Overexpression of miR-126-3p, miR-193b-3p and miR-92a-3p in various pairwise combinations reduced CCL2 secretion in an additive manner although the effects of different miRNA combinations were cell-specific in adipocytes and macrophages. These findings add novel insights into the TF and miRNA-mediated regulation of CCL2 production in human adipocytes.

In **Study II,** we identified adipocyte-expressed miRNAs altered by obesity that regulate adiponectin production. We found that miR-193b-3p increased adiponectin secretion and mRNA expression when overexpressed in human adipocytes. The expression of miR-193b-3p significantly correlated with adiponectin gene expression and insulin resistance measured by homeostasis model assessment of insulin resistance. We identified that miR-193b-3p binds directly to the 3'-UTR of negative adiponectin regulators such as nuclear transcription factor Y α and nuclear receptor interacting protein 1. This explains how miR-193-3p impacts adiponectin expression and secretion.

A WAT phenotype characterized by few but large fat cells (hypertrophy) is linked to metabolic complications. In **Study III,** we identified miRNAs that were significantly upregulated in hypertrophic WAT. Out of 15 identified miRNA, 10 were predicted to bind to early B cell factor 1 (EBF1), a recently described regulator of WAT morphology. Two miRNAs (miR-361-3p and miR-574-3p) bound directly to the 3'-UTR of *EBF1* and reduced *EBF1* expression alone as well as in a combinatorial manner. Transcription activity of EBF1 was negatively correlated with the expression of the miRNAs.

In **Study IV**, we identified 11 miRNAs that were differentially expressed between insulin sensitive and insulin resistance obese individuals. MiR-143-3p and miR-652-3p affected

insulin-stimulated glucose uptake either directly or indirectly affecting several insulin signaling regulators including Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), Protein Kinase, AMP-activated, Alpha 2 Catalytic Subunit (PRKAA2) as well as posttranslational phosphorylation of AKT2, AMP-activated protein kinase and insulin receptor substrate 1. Expression levels of miR-143-3p and miR-652-3p correlated significantly with insulin-stimulated lipogenesis suggesting that the levels of these miRNAs may also be of clinical importance.

In summary, we have defined the regulatory pathways for several miRNAs that are dysregulated in obese WAT and that may have a pathophysiological impact. Our studies describe the involvement of miRNAs in regulating the expression of CCL2, adiponectin, EBF1, ENPP1 and PRKAA2. These results provide a better understanding of the role of miRNAs in the regulation of WAT function in health and disease.

LIST OF SCIENTIFIC PAPERS

I. Kulyté A, **Belarbi Y**, Lorente-Cebrián S, Bambace C, Arner E, Daub CO, Hedén P, Rydén M, Mejhert N, Arner P. Additive effects of microRNAs and transcription factors on CCL2 production in human white adipose tissue. Diabetes. 2014;63:1248-58.

II. **Belarbi Y**, Mejhert N, Lorente-Cebrián S, Dahlman I, Arner P, Rydén M, Kulyté A. MicroRNA-193b Controls Adiponectin Production in Human White Adipose Tissue. J Clin Endocrinol Metab. 2015;100:E1084-8.

III. **Belarbi Y**, Mejhert N, Gao H, Arner P, Rydén M and Kulyté A. MicroRNAs-361-5p and -574-5p associate with human adipose morphology and regulate EBF1 expression in WAT.
Manuscript

IV. Dahlman I, **Belarbi Y**, Laurencikiene J, Pettersson AM, Arner P and Kulyté A. Comprehensive functional screening of miRNAs involved in human fat cell insulin sensitivity.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AGO	Argonaute
AMPK	AMP-activated protein kinase
APPL1	Adaptor Protein, Phosphotyrosine Interaction, PH Domain And Leucine Zipper Containing 1
AS160	Akt substrate of 160 kDa
ASCs	Adipose derived stem cells
ATGL	Adipose triglyceride lipase
ATMs	Adipose tissue macrophages
BMI	Body mass index
CCL2	Chemokine (C-C Motif) Ligand 2
CEBPα	CCAAT enhancer binding protein α
CIDEA	Cell Death-Inducing DFFA-Like Effector A
CoA	Coenzyme A
CREB5	cAMP-responsive element binding protein 5
DAG	Diacylglycerol
DEXA	Dual energy X-ray absorptiometry
EBF1	Early B cell factor 1
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
GLUT4	Glucose transporter type 4
HIF-1	Hypoxia inducible factor 1
HMW	High molecular weight
$HOMA_{IR}$	Homeostasis model assessment of insulin resistance
IL-6	Interleukin 6
IR	Insulin resistance
IRS-1	Insulin receptor substrate 1
LMW	Low-molecular weight
MAG	Monoacylglycerol
MARA	Motif activity response analysis
MAX	MYC-associated factor X
MCP-1	Monocyte chemotactic protein 1
miRISCs	MiRNA-induced silencing complexes
miRNA	MicroRNA
MMW	Medium molecular weight
NEFA	Non-esterified fatty acids
NF-YA	Nuclear transcription factor Y α
NRIP1	Nuclear receptor interacting protein 1
nt	Nucleotide
OIR	Obese insulin-resistant
OIS	Obese insulin-sensitive

PDE3B	Phosphorylation of phosphodiesterase 3B
PDPK1	Phosphoinositide Dependent Protein Kinase 1
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5-diphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein Kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PRKAA2	Protein Kinase, AMP-activated, Alpha 2 Catalytic Subunit
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)
RT-qPCR	Real time quantitative polymerase chain reaction
scWAT	Subcutaneous WAT
SP1	Specificity protein 1
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TAG	Triacylglycerides
TF	Transcription factor
TNF-α	Tumor necrosis factor α
TRN	Transcription regulatory network
UTR	Untranslated region
VEGFA	Vascular endothelial growth factor A
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WHR	Waist/hip circumference ratio

1 INTRODUCTION

1.1 OBESITY

Obesity prevalence has grown rapidly in the last decades and is a worldwide health and society problem (1). In 2014, the estimated number of overweight and obese adults was more than 1.9 billion, of which 600 million fulfilled the WHO criteria for obesity (2). Initially, this was mainly a concern in high-income countries however now it also poses a problem to middle and low-income countries. Obesity belongs to the cluster of medical conditions termed metabolic syndrome which is defined by visceral adiposity, hypertension, dyslipidemia and high blood pressure (3). The metabolic syndrome is associated with a number of complications such as type 2 diabetes (T2D), cardiovascular disease and cancer to mention a few (3). Since the 1980s, the prevalence of T2D has risen from 108 to 422 million in 2014 which parallels the increase in obesity (4). This constitutes a large financial burden to society and so far the counter-measures have been insufficient (5,6).

From an evolutionary perspective the human body is set to conserve calories in order to withstand periods of limited food supply. Our modern society provides almost unlimited availability to calorically dense foods and leads a sedentary lifestyle. According to the thrifty gene hypothesis, it is our evolutionary conserved genome that explains the resulting increase in obesity prevalence (7,8). In simple terms, development of obesity is primarily explained by an imbalance between energy-intake and energy expenditure. Early onset development of obesity e.g. during childhood increases the risk to become overweight or obese in adult life. Low economic status and education level are additional risk factors (9). Contrary to common belief, genetic mutations in selected genes do not contribute to common forms of obesity (10).

Obesity can be defined in numerous ways such as body mass index (BMI), waist circumference, waist/hip circumference ratio (WHR) and body fat percentage. The most frequently used method in research is BMI due to applicability in large-scale epidemiological studies; it is quickly measured and relatively reproducible. BMI is calculated by dividing the individual's weight in kilogram with the square of their height in meters (kg/m^2). The categories reflects weight related problems; the division of classes are shown in Table 1 (11). One should mention that BMI scaling is applied for white Caucasian adults and does not include elderly, children or well-trained individuals. BMI classification is also arbitrary for other ethnic groups, especially for Asian populations thus highlighting the need to develop a more suitable classification system for other ethnic groups (12). Waist circumference is a better indicator for abdominal fat accumulation and risk predictor of obesity-related morbidities. The cut-off values for increased relative risk of disease is <102 cm for men and <88 cm for women (11). A more accurate assessment of body fat requires more advanced equipment or time than BMI and WHR. It requires a specialist, thus cannot be self-assessed, performing measurements such as skin fold thickness, bioelectric impedance, air/water

displacement, whole body dual energy X-ray absorptiometry (DEXA) and computerized tomography and magnetic resonance imaging.

Table 1. BMI categories

Categories	BMI (kg/m^2)
Underweight	>18.5
Normal weight	18.5-24.9
Overweight	25.0-29.9
Obese	<30.0

Weight reduction can only be achieved by altering the equilibrium between energy-intake and energy expenditure. However, calorie restriction and exercise is very demanding for the individual and hard to maintain (6). To date, the best method for weight reduction is bariatric surgery. There are several techniques such as gastric bypass, gastric sleeve and gastric banding. Gastric banding results in less pronounced weight reduction and has therefore become less used (13). Gastric bypass rapidly improves the metabolic profile and glucose control, even prior to weight loss. This is achieved via an altered hormonal release in response to food intake, e.g. increased levels of glucagon like peptide -1 and peptide YY but reduced ghrelin levels. Altogether these changes increase insulin release and the sense of satiety (14). However the main disadvantage of bariatric surgery is the highly expensive costs and is therefore not a sustainable solution for treating obesity.

1.2 ADIPOSE TISSUE AND ITS FUNCTIONS

In terms of size, white adipose tissue (WAT) displays the most pronounced inter-individual variations. In extreme states, humans may have fat ranging from a few percent up to 50 % of the total body weight. WAT is a loose connective tissue where almost all volume is consisting of adipocytes. Adipocytes are surrounded by the stromal vascular fraction (SVF), which is composed of progenitor cells, fibroblasts, leucocytes and endothelial cells. WAT in humans can be divided into two main compartmental sites, visceral and subcutaneous. Visceral fat is located in the abdominal cavity and is known to be more metabolically active. This region releases non-esterified fatty acids (NEFAs) directly into the portal vein that transports blood to the liver and pancreas, two organs sensitive to lipotoxicity. Subcutaneous fat is located underneath the skin and serves as thermal isolation and a mechanical shield for the body. However, its most important function is to store and release energy. Excess energy is accumulated in the form of triacylglycerides (TAG). During energy deficiency TAGs are hydrolyzed into NEFAs and glycerol, a process termed lipolysis (detailed in section 1.2.1.1). Last but not least, WAT also serves as an endocrine organ, secreting a large number of factors and proteins named adipokines (detailed in section 1.5).

1.2.1 Adipocytes

Adipocytes have a large lipid droplet that constitutes >95% of the cell volume. In WAT, adipocytes constitute at least 90 % of the tissue volume and about 10 % of the adipocytes are renewed every year (15). Adipocytes are recognized as critical regulators of whole-body metabolism primarily through lipolysis and NEFA synthesis (lipogenesis), in the following section a more comprehensive description will be given.

1.2.1.1 Lipolysis

Lipolysis is the enzymatic breakdown of TAGs. One TAG is broken down to one glycerol molecule and three NEFA molecules and this is achieved in three steps:

I. TAG is hydrolyzed to diacylglycerol (DAG) by adipose triglyceride lipase (ATGL)
II. DAG is converted to monoacylglycerol (MAG) by hormone sensitive lipase (HSL)
III. The breakdown of MAG to NEFAs and glycerol is accomplished by both monoacylglycerol lipase (MGL) and HSL.

ATGL is the rate-limiting enzyme of lipolysis. A small part of NEFAs remain in the adipocyte for re-esterification but first NEFAs are activated by the addition of coenzyme A (CoA). Most of the NEFAs are released into the circulation bound to albumin and can be used through β-oxidation by other organs such as muscles, the pancreas, heart and liver to produce energy. In the liver, NEFAs are also used for synthesis of TAG or very low-density lipoprotein (VLDL). The phosphorylation of the glycerol molecule generated in adipocytes does not occur due to the low expression of the enzyme glycerol-3-kinase. Although it is phosphorylated to glycerol 3-phosphate in the liver where it can further be used for lipogenesis (16).

Lipolysis occurs constantly at a low rate which is termed basal (spontaneous) lipolysis. Lipolysis is regulated by numerous factors but in humans the most essential are catecholamine's, natriuretic peptides (both pro-lipolytic) and insulin (anti-lipolytic). The effects of catecholamine's are somewhat complex as they induce lipolysis through β adrenergic receptors and inhibit it via α2-A adrenergic receptor (16). Tumor necrosis factor α (TNF-α) also belong to the more important lipolysis regulators (16). Other factors that also impact lipolysis are age, gender, nutrition and physical activity. Lipolysis is altered in different metabolic conditions; in obesity basal lipolysis is increased whereas stimulated lipolysis is attenuated. The end result is elevated circulating levels of NEFAs which are normalized upon weight reduction.

1.2.1.2 Lipogenesis

Liver is the primary tissue for *de novo* lipogenesis, synthesis of NEFA (17). This pathway is mainly stimulated by elevated glucose and insulin concentration in the blood and consequently augmented glucose uptake in hepatocytes. Intracellularly, glucose is phosphorylated to glucose-6-phosphate and processed further to acetyl-CoA, which is an important intermediate substrate for NEFA production. Glucose uptake by WAT is minor in

comparison to whole body uptake, constituting approximately 5 – 10 % (18). Instead the main source for WAT lipogenesis originates from diet fat for synthesis of TAG. In the blood TAGs are transported in lipoproteins such as chylomicrons or VLDL. On the cell membrane of adipocytes, lipoprotein lipases attach to VLDL and cleave TAGs into NEFAs thereby allowing the adipocyte to take up NEFAs through fatty acid transporter or passive diffusion. Once inside the cell, the enzyme fatty acyl-CoA synthetase catalyzes the formation of fatty acyl-CoA by addition of CoA to NEFAs. The formation of TAGs can now occur; three NEFAs are assembled with one glycerol 3-phosphate. The uptake of glucose and TAGs from VLDL is highly dependent on insulin. In obesity, the insulin response is reduced meaning that the storage of fat is attenuated. This results in ectopic storage of fat in non-adipose tissues causing lipotoxic effects.

1.3 ENDOCRINE FUNCTION OF ADIPOSE TISSUE

1.3.1 Insulin

Insulin is the most important hormone for regulating energy homeostasis and its most prominent effect on glucose metabolism are exerted on the muscle, liver and WAT (18). In WAT insulin has a central role in inhibiting lipolysis and stimulating processes such as adipocyte differentiation, lipogenesis, glucose and NEFA uptake as well as NEFA re-esterification.

1.3.1.1 Insulin signaling

Insulin is controlled at numerous levels and more than 100 genes are involved in the regulation as well as numerous phosphorylation events (19,20). There are two insulin-signaling pathways:

1. Phosphatidylinositol-3 kinase (PI3K) dependent pathway
2. Non-canonical CBL-TC10 pathway

The insulin signaling cascades are initiated when insulin binds to the insulin receptor extracellularly subsequently activating intracellular auto-phosphorylation of tyrosine residues. The phosphorylated parts acts as docking sites for insulin receptor substrate (IRS) proteins leading to the tyrosine phosphorylation of IRS. The IRS proteins stimulate PI3K which catalyzes the conversion of phosphatidylinositol-4, 5-diphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). The membrane-bound PIP3 binds and activates phosphoinositide dependent protein kinase 1 (PDPK1). In turn, PDPK1 together with mammalian target of rapamycin complex 2, phosphorylates AKT at different sites thus activating it. The activation of AKT is central for glucose uptake and regulation of lipolysis. For glucose uptake, AKT catalyzes phosphorylation of AKT substrate 160 (AS160). Phosphorylated AS160 is inactivated thereby allowing the increased translocation of GLUT4-containing vesicles to the membrane and facilitating glucose uptake. The activated AKT will mediate inhibitory effect on lipolysis by phosphorylation of phosphodiesterase 3B

(PDE3B). PDE3B ameliorates degradation of cAMP to 5'AMP that decreases phosphorylation events of PKA and HSL, thus their activity.

Within the CBL-TC10 pathway, insulin receptor binds to the proteins APS, C-cbl associated protein and CBL followed by phosphorylation. Activated CBL triggers docking of CRK guanine nucleotide exchange factor, C3G, to the plasma membrane, which further stimulates small GTPase TC10 and GLUT4 translocation. Numerous genes and phosphorylation events control the recycling process of GLUT4. Detailed description of the signaling events linked to GLUT4 have been extensively reviewed (19).

1.3.1.2 Insulin resistance

Insulin resistance (IR) is a pathological condition where cells display a reduced ability to respond to insulin. This results in augmented glucose levels in the blood and is compensated by increased production of insulin from the pancreas. With time, the β-cells in the pancreas will lose their ability to produce insulin in sufficient amounts and the peripheral tissues gradually increase IR. Glucose levels therefore increase, leading to hyperglycemia and the development of T2D. IR occurs in several organs but the complete mechanistic pathology is not fully understood. From a WAT perspective, the increased lipolysis in obesity augments NEFA levels in the blood with deleterious effects by promoting lipid storage in muscle, liver and pancreas (21). In muscle, NEFAs will impair glucose uptake by activation of protein kinase C (PKC) θ, which will prevent insulin to stimulate muscle glucose uptake by reducing the intracellular GLUT4 translocation. NEFAs also reduce hepatic insulin signaling through their activation of PKCε. The activated kinase will also limit insulin-stimulated glycogen synthesis but the lipid synthesis remains constant. In addition, by increasing the hepatic levels of acetyl-CoA, NEFAs will activate hepatic gluconeogenesis causing increased release of glucose into the circulation (18).

1.3.2 Adipokines

It is well established that WAT is an endocrine organ, secreting a large number of factors termed adipokines. These are released to the bloodstream and affect processes linked to glucose and fat metabolism in muscle, liver and brain. Adipokines also have an important function for the communication between the various cell types within the tissue, interacting in an auto-paracrine fashion. The cross talk is essential for the regulation of processes such as adipogenesis, proliferation, vascularization, inflammation, stress, metabolism and tissue structure (22). Due to the great numbers of adipokines, only the most relevant for this thesis will be described in the following section.

1.3.2.1 Adiponectin

Adiponectin was first discovered two decades ago and since then it has been intensively studied (23). Adiponectin is a unique adipokine because of its anti-inflammatory effects. In obesity, plasma levels of adiponectin are markedly reduced compared to lean individuals (24). Adiponectin consists of 244 amino acids; the monomeric form is 28 kDa. In the

circulation adiponectin is only found in three isoforms where the monomer is associated to structures linked with disulphide bonds:

I. Trimers, low-molecular weight (LMW), 67 kDa
II. Hexamer, medium molecular weight (MMW), >120 kDa
III. Higher order multimers, high molecular weight (HMW), > 300kDa

The oligomeric isoforms are not equally potent, though, HMWs are believed to be the most active form exerting the insulin-sensitizing effects (25,26). In addition, there are several post-translational modifications that contribute to diverse multimerization and function (27). A central step for these biological effects is mediated through phosphorylation of AMP-activated protein kinase (AMPK) (28,29). This step is facilitated by another protein linking the ligand and the ADIPOR1/2 receptor, adaptor Protein, Phosphotyrosine Interaction, PH Domain And Leucine Zipper Containing 1 (APPL1) (30). Adiponectin mediates its effects by binding to two receptors, ADIPOR1 and ADIPOR2, which are expressed in muscle and liver. In muscle, adiponectin enhances NEFA oxidation as well as glucose transport by promoting translocation of GLUT4 (28). In the liver, adiponectin decreases gluconeogenesis and improves insulin sensitivity (31). Consequently, mice lacking adiponectin develop IR (32).

1.3.2.2 Chemokine (C-C motif) ligand 2

Chemokine (C-C Motif) Ligand 2 (CCL2) also called monocyte chemotactic protein 1 was initially identified as a chemokine secreted by macrophages as well as endothelial cells, and is a key factor for the recruitment of monocytes from the circulation. Once in the tissue, monocytes will differentiate into macrophages by activation from various actors e.g. lipopolysaccharides, interferon γ and interleukin 6 (IL-6). Murine models have revealed that CCL2 is also secreted from adipocytes and is an important player in the inflammatory response due to its chemoattractant action on macrophages in obese WAT. When CCL2 is disrupted in mice on a high fat diet, they get resilient to obesity-induced IR and display features such as improved insulin sensitivity, increased macrophage response and decreased hepatic TAG content (33). Similar results are obtained when knocking down the CCL2 receptor; C-C motif chemokine receptor-2 (34).

There are also indications that this cytokine may contribute to the pathogenesis of IR by disrupting glucose uptake. This was demonstrated in 3T3-L1 murine cells treated with CCL2 where glucose uptake was significantly impaired (35). Other cells within WAT may also contribute to the production of CCL2, in fact the secretion is significantly larger from SVF compared to isolated adipocytes (36). In obese humans, visceral fat have a significantly higher production of CCL2 compared to subcutaneous fat but when adjusting for the number of macrophages the difference is attenuated (36). Altogether, evidence indicates that CCL2 is involved in obesity-related disorders such as adipose tissue inflammation and might be a link to IR.

1.4 PATHOGENSIS MECHANISMS OF ALTERED WAT IN OBESITY

WAT is a highly plastic organ with large variations in size and the adipose tissue remodeling is an ongoing process that is accelerated in obesity. This is characterized by functional dysregulation of WAT, such as altered release of adipokines and NEFAs. There are several different pathways involved in these changes such as increased inflammation and immune cell infiltration, reduced vascularization, increased extra cellular matrix (ECM) production and altered morphology of WAT. The chronological order of the mentioned events are debated but altogether increase the risk for obesity-associated complications (37).

1.4.1 Inflammation

Adipose tissue contains a distinct population of immune response cells, macrophages and lymphocytes. In obesity, there is an increased infiltration of macrophages, which are important regulators of WAT inflammation. The chronic low-grade inflammation in WAT is suggested to link increased adiposity to reduced insulin sensitivity by attracting more pro-inflammatory cells. Consequently, there will be increased levels of cytokines such as TNF-α and IL-6, both inducing lipolysis in adipocytes. CCL2 is also induced, and acts as an important chemoattractant factor in WAT, as described previously in section 1.3.2.2.

Adipose tissue macrophages (ATMs) are essential for the dynamic state of adipose tissue, allowing it to adjust to changes in energy expenditure and intake by increasing or reducing fat mass. ATMs are classified into two main phenotypes "M2" and "M1". The role of M2 ATMs is to clear the tissue from lipids, cellular debris and apoptotic cells (37) and to preserve a normal function of adipocyte. In contrast, M1 ATMs are overrepresented in the obese state and are characterized by increased release of cytokines (TNF-α, IL-6 and CCL2). In severe obesity, M1 macrophages aggregate around necrotic adipocytes forming crown-like structure which reflects an inflammatory state (22,37). Recently, the dichotomous categorization into M1 and M2 has been suggested to be oversimplified as the phenotypic range of ATMs appears to be considerably more complicated (38).

1.4.2 Morphology

The morphology of WAT, i.e. the size and number of fat cells, is an important aspect of obesity, related to dysfunction and fat cell metabolism. At any given amount of fat mass, the tissue can be composed of few large fat cells (hypertrophy) or numerous small cells (hyperplasia) (39). This hypothesis was first coined in the sixties but it has subsequently been shown that hyperplasia is more beneficial from a metabolic point of view whereas hypertrophy is associated with increased adipose inflammation, lipolysis, IR and risk of type 2 diabetes (40,41). The mechanisms that promote the development of these opposing phenotypes are not clear.

1.4.2.1 Early B cell factor 1 as a regulator for adipocyte morphology

The transcription factor Early B cell factor 1 (EBF1) has been recently demonstrated as an important factor regulating WAT morphology (42). EBF1 was originally identified as an

essential player for B-lymphocytes maturation (43). Later it was shown to be involved in the development of other cell types including adipocytes (44). In adipogenesis, EBF1 is participates in the early adipocyte commitment and has shown to bind to peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) (45). In EBF1 knock out mice, there is a pronounced difference in size of the mice. EBF1 deficient mice are significantly smaller and display an increase in bone formation and reduced adipose tissue (46). In recent years, a wider understanding of the role of EBF1 in adipocytes has been reached. EBF1 was identified as a crucial player in insulin signaling where EBF1 loss in fat cells demonstrated a reduced insulin stimulated glucose uptake and lipogenesis. Inflammatory release of cytokines was also affected by EBF1 knockdown through toll-like receptors. Decreased EBF1 expression in mice results in WAT hypertrophy (42). Attenuated EBF1 activity and protein expression in human subcutaneous WAT (scWAT) is associated with several parameters of the metabolic syndrome (47). However, regulatory mechanisms of EBF1 activity are yet not defined. So far, only TNF-α has been suggested as an upstream regulator of EBF1 (42).

1.4.3 Extracellular matrix composition

Another crucial element in WAT remodeling is the extracellular matrix (ECM) (48). ECM builds up WAT architecture and contains structural proteins such as collagens in addition to adhesion proteins including fibronectin, laminin, elastin and proteoglycans. ECM is a non-cellular portion of WAT surrounding the adipocyte like a web and the plasticity of ECM is believed to affect the morphology phenotype. It has been suggested that a relaxed ECM is better for a "healthy adipose tissue", allowing adipocytes to grow and be dynamic upon nutritional changes. Conversely, an inflexible matrix would result in restricted adipocyte growth, ectopic fat accumulation and hence, increased risk of disease. The ECM homeostasis (balance between production and degradation) is disturbed in obesity and the damaging effect comes from increased synthesis of ECM components or impaired degradation resulting in fibrosis (49). There are currently two hypotheses on the chronological order of fibrosis and IR. The first suggests that WAT has a limited expansion capability and that fibrosis induces hypoxia which causes inflammation (50). The second theory proposes that WAT growth induces inflammation that in turn leads to fibrosis (51). Regardless of the order, a vicious cycle is created which contributes to an inflammatory condition.

1.4.4 Vascularity

Sufficient blood flow favors adipose tissue formation and the basis for this is angiogenesis. Although WAT is in general described as poorly vascularized when compared to other tissues, a dynamic vascular system is critical for WAT expansion and is considered to be rate limiting for WAT expansion (37). In addition, the obese state is associated with reduced supply of oxygen and density of vascularization, a condition termed hypoxia (52,53). Although recently, the first human study reported conflicting data, showing increased adipose tissue oxygen tension in obese compared with lean individuals (54). The theory behind this is that inadequate vascularization in adipose growth and/or increased adipocyte

size instead of number increases diffusion distances for oxygen. In hypoxia, there is an induction of the transcription factor (TF) hypoxia inducible factor 1 (HIF-1), which subsequently increases genes such as *IL-6*, vascular endothelial growth factor A (*VEGFA*), GLUT-1 and reduction of adiponectin (55). HIF-1 also induces ECM genes and indirectly influences fibrosis (50). Altogether, this suggests that hypoxia is involved in the pathogenesis of obesity by disrupting ECM homeostasis and inflammation response. This illustrates the complexity of WAT and its function.

1.5 GENE REGULATION

The central dogma of molecular biology postulates that genetic information is from DNA via mRNA to proteins. It is mechanistically quite well understood how genes are transcribed and how the mRNAs are translated into amino acid chains. Of course, gene regulation is much more complex than this three-step model and cellular function can be controlled at different levels involving numerous factors and post-translational events such as phosphorylation, methylation, acetylation, attachment of peptides, etc. The interaction among regulatory protein, transcription factors, RNA-molecules create complex regulatory networks. In addition a new set of post-transcriptional modifiers has been highlighted in the last decade termed microRNA (miRNA). They participate in almost every cellular process and are recognized as fine-tuning actors.

1.5.1 MicroRNA

MiRNAs are single stranded non-coding RNAs that belong to the RNA-interference class of regulators known to inhibit gene expression. In general, miRNAs are estimated to regulate up to 60 % of the genes in the human genome (56). A wide range of miRNAs have been reported to participate in obesity and the development of the metabolic syndrome. More specifically in adipocytes, miRNA are important for adipogenesis, fat cell development and regulation of the metabolic and endocrine function as reviewed (57). The expression of miRNAs is influenced by the state of health, and in obesity numerous miRNAs have been demonstrated to be dysregulated in adipose tissue and inflammation but also other tissues important for metabolism as reviewed (58). However, only a smaller part of expressed miRNAs have been verified experimentally in humans and even less detailed regulatory pathways have been described so far.

1.5.1.1 Biogenesis of miRNA

MiRNAs originate from the precursor molecules pri-miRNA, a double stranded RNA transcripts folded in a hairpin structure. The generation of miRNA is performed in two main steps. In the nucleus pri-miRNA is cropped to a 70-nucleotide (nt) pre-miRNA by the enzyme Droscha and further transported out to the cytosol. Here, pre-miRNA is cleaved by the enzyme Dicer to an approximately 20 nt miRNA duplex, one strand with 3' end and a 5' terminus strand. Generally, one strand is selected as mature miRNA whereas the other is degraded. Sometimes both strands of the duplex are used, functioning as mature miRNA. The single stranded miRNA is 18-23 nt of length and is fused together with ribonucleoprotein

from the Argonaute (AGO) family forming a complex named; miRNA-induced silencing complexes (miRISCs). Apart from the canonical miRNA biogenesis depicted in figure 1, there is also an alternative passage described which bypasses Droscha or Dicer processing (59). The miRISC guides the miRNA to the specific target by complementary binding to the 3'-untranslated region (UTR) of target mRNAs. In the present thesis the research emphasis is on the systemic effect generated by miRNAs that is a squared area in figure 1.

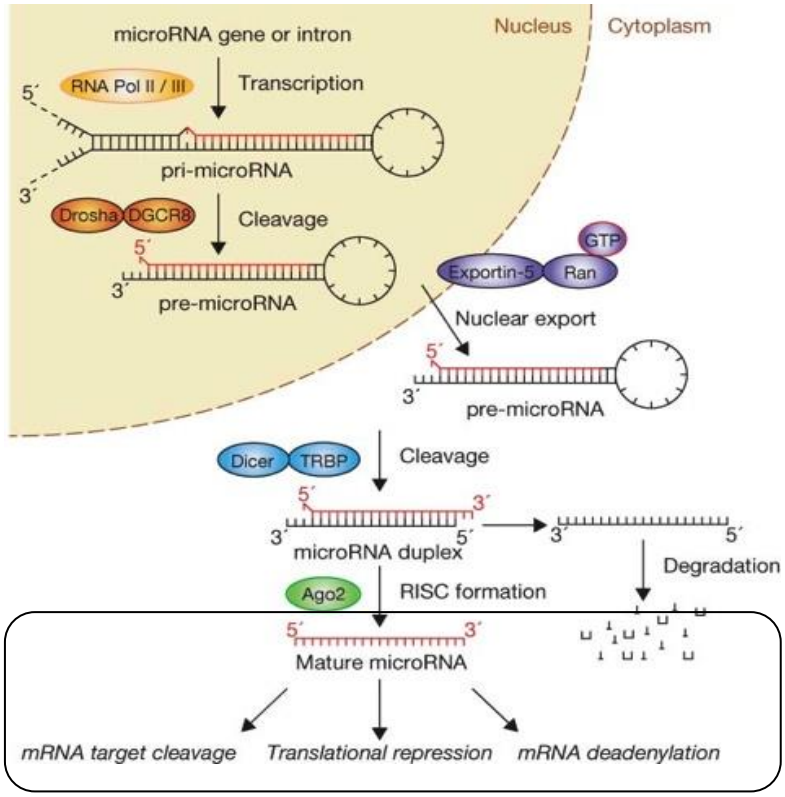

Figure 1. The canonical pathway of microRNA processing. Source: Nat Cell Biol 11:228-34 (2009) (60). Printed with minor changes and with permission from the publisher.

1.5.1.2 Silencing effects

The interaction of base pairing of 2-8 nt needs to occur in the miRNA-mRNA association, also called seed region. The interface between miRNA and the gene enables the AGO-proteins in the miRISC to repress the translation or induce mRNA degradation (61). The fate is determined by how well the miRNA binds to the target sequence, in other words, perfect or imperfect pairing results in various suppressions actions. Translational repression is mediated through imperfect base pairing. The exact mechanism is still not fully understood, yet data suggest that repression action occurs at initiation and elongation step (62–64). On the other hand, perfect base pairing causes in general mRNA disruption, the mechanistic feature differs between plants and animals (65). In animals, the removal of the poly (A) tail causes the mRNA strand to be more vulnerable for exonucleolytic degradation. In plants, the mRNA degradation is endonucleolytically cleaved by AGO (66). In contrast, there have also been indications that miRNA can activate translation although the mechanism is poorly described (67,68).

The effect of miRNAs on target genes can be executed in various ways, either directly or indirectly. The direct actions of miRNAs mean the interaction of the miRNA with the gene and thereby having repressive effect. Indirect involvement of miRNA allows the interaction of different components such as TFs, co-transcription factors and genes. Together, various signaling cascades creates a network with a few or several nodes (57). Identification of regulatory pathways is therefore a challenging task in the miRNA research. In figure 2 the different possible interplay between miRNAs and TFs are illustrated.

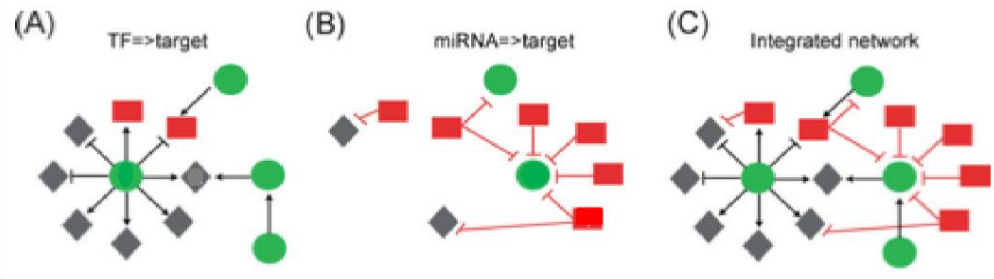

Figure 2. The interplay between microRNA and transcription factors. Different types of regulatory networks involving miRNAs and TFs can be distinguished. (A) Transcription regulatory networks that consist of protein-DNA interactions between TFs and the cis-regulatory DNA regions of their target genes (TF → target). (B) Post-transcription regulatory networks that consist of RNA–RNA interactions between miRNAs and the 3'-UTR of their target mRNAs (miRNA → target). (C) Both types of networks can be combined into gene regulatory networks. Source: Bioessays. 2009;31:435-45 (69). Printed with minor changes and with permission from the publisher.

1.5.1.3 Identification of miRNA targets

There are a number of bioinformatics tools available to identify predicted targets of miRNA, the most common being miRanda, PicTar, PITA, TargetScan and RNA22. An extensive search can be performed on the platform miRWalk, which combines the information from separate database and miRNA target prediction programs (70,71). In general, the emphasis is on the miRNA-mRNA interaction 3'-UTRs, although emerging evidence imply the binding of 5'-UTRs, coding DNA sequence and/or non-coding RNAs (72). The 3'-UTR may contain multiple targeting sequences and other regulatory elements due to imperfect base pairing of miRNAs. Furthermore, one miRNA can bind a large number of genes and conversely one gene can be a target for multiple miRNA, see figure 2 (69). To comprehend the functions of miRNA in complex biological processes, it is important to experimentally assess the functional relevance of the predicted targeting site(s).

2 HYPOTHESIS AND AIM

2.1 GENERAL HYPOTHESIS AND AIM

The overall hypothesis for this thesis is that miRNAs influences numerous factors regulating different aspects of adipocyte physiology thereby contributing to WAT dysfunction and development of IR. In four studies presented in the thesis, this has been investigated from different angles with the general aim to characterize dysregulated miRNAs in WAT, and link them to pathological pathways causing inflammation and IR.

2.2 SPECIFIC AIM FOR THE STUDIES

I. Extend and validate CCL2 regulatory network consiststing of obesity-dysregulated miRNAs and TFs in human adipocytes and macrophages.

II. Determine if obesity-dysregulated miRNAs are involved in adiponectin regulation in human fat cells.

III. Map expression of miRNAs associated with WAT morphology and their impact to EBF1 regulation.

IV. Characterize miRNA expression profile in insulin resistant fat cells and elucidate their regulatory pathways affecting insulin signaling.

3 REMARKS ON THE METHODS

In this section I, will give a brief introduction of the techniques used in this thesis and describe their strengths and weaknesses. A more detailed explanation of the methods is found in the corresponding papers.

3.1 SUBJECT CHARACTERISITICS AND WHITE ADIPOSE TISSUE SAMPLES

3.1.1 Cohorts

Ethical approval was obtained for all studies and followed the statutes of The Declaration of Helsinki. All subjects included in the respective cohorts gave their informed written consent prior to participation. Three first studies are based on the same cohort, also referred to as cohort 1. Cohort 1 consists of 30 obese and 26 non-obese healthy women, see table 2 for clinical parameters (73). Study IV comprises 18 obese insulin-resistant (OIR), 21 obese insulin-sensitive (OIS) and 9 lean women, cohort 2. The characteristics of this group are described in detail in the paper.

Table 2. Clinical characteristics of Cohort 1

Clinical Parameter	**Non-obese** (n=26)		**Obese** (n=30)	
Age (years)	23-72	*(43±14)*	27-66	*(43±10)*
Weight (kg)	53.6-92.8	*(67.6±8.5)*	65.8-153.0	*(111.9±22.1)*
BMI (kg/m^2)	20.4-27.7	*(24.2±1.8)*	30.5-53.0	*(41.0±6.8)*
Waist circumference (cm)	69.5-113.0	*(85.2±9.2)*	99.0-147.0	*(121.7±13.9)*
Body fat (%)	22.5-38.9	*(30.3±3.7)*	41.0-73.3	*(55.5±9.7)*
Fat cell volume (pL)	317.0-877.0	*(501.6±153.8)*	511.0-1349.0	*(899.7±208.0)*
Total adipocyte number (10^{10} cells)	2.1-8.3	*(4.8±1.5)*	3.5-13.8	*(7.9±2.6)*

Values are shown as range *(mean ± standard deviation)*

3.1.2 White adipose tissue samples

All subjects underwent a fine needle biopsy from subcutaneous abdominal WAT under local anesthesia. The adipocytes were isolated from the fat sample and the total number of adipocytes in the body was estimated, by dividing the total weight of body fat by mean adipocyte weight (15). The function of adipocytes is not disturbed by the biopsy procedure (74).

3.1.2.1 Adipocyte morphology

In study III, we compared the expression of miRNAs between hyperplasia and hypertrophy. In order to determine the morphology of the tissues, we used a curve fit relationship between adipocyte volume and the relationship between weight and length (BMI). The difference between observed and expected adipocyte volume obtained from the fitted curve reflects adipose morphology, where a positive value defines hypertrophy and a negative number hyperplasia (75).

3.1.3 Systemic insulin sensitivity

Insulin sensitivity was determined by homeostasis model assessment of insulin resistance ($HOMA_{IR}$). Fasting plasma insulin and glucose is measured and $HOMA_{IR}$ is calculated with following formula:

$$HOMA_{IR} = \frac{\text{Fasting plasma insulin (mU/L)} * \text{Fasting plasma glucose (mM/L)}}{22.5}$$

The cut off-value for IR was >2. This method is well established and frequently used and shows a good correlation with measures obtained using the golden standard hyperinsulinemic euglycemic clamp (76).

3.2 CELL CULTURE SYSTEMS

3.2.1 Primary cells

In this thesis, almost all experimental data was generated in *in vitro* differentiated preadipocytes isolated from SVF of human WAT. This technique is well established and the cells have been characterized in detail before (77,78). The primary cultures display higher inter-individual variations compared to classical cell lines such as the murine 3T3-L1. This disadvantage can be avoided by using adipose derived stem cells (ASCs). They are obtained from one donor and are possible to expand through several passages. The common feature of ASCs and SVF-derived adipocytes is that they both have multiple lipid droplets. The most *in vivo*-like model is mature fat cells having one large fat droplet. The primary use of mature fat cell is for determination of adipokine secretion and assessment of lipogenesis. The main disadvantages with mature fat cells are that they float, fragile, difficult to handle and can only be kept in cultured medium for a maximum of a few days, thereby limiting the possibility of performing mechanistic studies.

3.2.2 Immortalized cell line

Immortalized cells were used as a complementary option to primary cells. The murine cell line 3T3-L1 was used for plasmid transfections, because it is an easier model system and well accepted cell-carriers for reporter assays. These cells are convenient because they are robust, less sensitive than primary cells, expand quickly, cost-effective and overall easy to handle. On the other hand the drawback is that the cell cycle machinery has been manipulated and therefore the risk for spontaneous mutation is high. Every passage is also identical thus biological variation is low in addition to originating from another species. As mentioned in the papers, these cells were used for mechanistic experiment investigating if miRNA bind to the 3'-UTR sequence of a gene. Therefore the species differences might be of less importance, because we examined the physical binding.

In paper I, THP1 cell line was used to study monocyte/macrophage cells, THP1 cells are derived from peripheral blood of a leukemia patient. Macrophages are flexible and assorted cells type. Depending on stimuli and tissue localization the differentiation mechanism will

vary and consequently trigger different polarization. In study I, the monocyte was differentiated with phorbol myristate acetate according to protocol into a non-polarized type (M0). This may not reflect their in vivo presence in WAT, and thus to get a more analogous environment co-culturing can be applied.

3.3 DIFFERENCES IN SPECIES

Within the metabolic research field it is highly appreciated to use animal models in order to implement specific functions in a complete and whole biological system. It is more common to study adipose tissue in mice, and subsequently follow-up in human samples, usually due to ethical reasons and difficulties of obtaining samples. Although it might be more favorable to do the opposite e.g. first generating hypotheses based on human cohorts, then perform mechanistic studies in *in vitro* systems and verify in animal models. This is due to the inter-species differences and the history of demonstrating pathological pathways in animals which has later been shown to be irrelevant in humans (79). The most obvious dissimilarity between human and rodents is the physiology and the distribution of brown adipose tissue and WAT. Human depots do not have a correspondence in rodents, e.g. the human visceral fat hardly exists in mice. Instead epididymal fat of male rats is used as a proxy for visceral fat (80).

There is regional variation in the production of adipokines by adipose tissue in human vs. rodents. Several adipokines haves also been proven to exhibit contrasting roles. Adipsin and resistin are two examples, in addition to TNF-α that is not released into circulation as seen in mice (79,81). TNF-α is known to regulate adipocyte lipolysis although through different signaling pathways between species (82). There are also pathways for lipolysis regulation in humans that does not existing in rodents. Two examples of regulatory pathways found in only man is the *α2* adrenergic receptor signaling negatively regulates lipolysis whereas natriuretic peptides are found to markedly increase lipolysis. There are also few examples of gene-isoforms e.g. IRS-3 which undergoes tyrosine phosphorylation in rodent adipocytes but no functional gene or protein is found in humans (83). Another example is HSL, there are two isoforms in humans and one is unique displaying pathophysiological importance in insulin-resistant conditions, while only one form exists in mice (84).

It is common to use obese models when performing research; the most recognized is leptin deficient mice (ob/ob) and disrupted leptin receptor mice (db/db). These mice represent an extreme state and leptin associated condition in humans is uncommon. Except for the obvious physiological differences between species this postulates why effects may differ between murine and human studies.

3.4 MIRNA STRATEGIES

3.4.1 Expression profiling

The initial technical tool for this thesis is gene microarray, a methodology that enables global miRNA expression profiling. Results in papers I-III are based on the same array comparing the miRNA expression in WAT from lean and obese patients. For paper III, only

a subset of subjects were included and the array data was therefore reanalyzed based on the hypothesis. In study IV, we hypothesized altered miRNA expression between OIS and OIR and therefore an array was performed from cohort 2. The main advantages of microarray are the relatively low cost and small amount of RNA required without amplification. Microarray is a small chip containing several probes for each sequence of the genome but the discovery is limited by the annotated miRNAs on the chip. The workflow of the procedure starts with sample preparation implying isolation of RNA, conversion to cDNA and further the transcripts are labeled with fluorescent dyes. The sample material is put on the array for hybridization, washed and a laser registers light intensities. The emission is translated to expression of sequence by computational analyses and validation of the data is done by with real time quantitative polymerase chain reaction (RT-qPCR). Both the methods, microarrays and RT-qPCR, will reveal the relative difference in expression and not the absolute quantification. RNA-sequence is a more extensive technique, supplying with the absolute quantification and nowadays is considered as golden standard for translational profiling (85).

3.4.2 Targets

3.4.2.1 Bioinformatics approach

Target identification of miRNA is usually a challenging work. Computational tools are extensively used and there are numerous commercial available databases, mentioned in section 1.6.1. The prediction is calculated with algorithms based on base- pairings between the miRNA and mRNA seed sequences, conservation among species and thermodynamic stability of the miRNA-mRNA interaction (86). Due to the great number of false-positives, functional validations are necessary *in vitro* and *in vivo*.

3.4.2.2 Validation of targets

Reporter assay in vitro was used to validate binding of miRNAs to its predicted mRNA targets and is an established method used to determine miRNA-mRNA interaction. In brief, the 3'-UTR sequence of target gene is inserted downstream of reporter gene in a DNA vector. We used dual Firefly/Renilla luciferase reporter that were obtained from Gene Copoeia. Activity of firefly luciferase is used for the detection of miRNA binding, e.g. when miRNA binds to its predicted target sequence, the luciferase activity is reduced and luminescence is decreased. Renilla luciferase gene in the same vector is used as an internal control for normalization of Firefly luciferase activity. Transfections were performed in murine 3T3- L1 cells using lipofection-based transfections agents as described in the papers. Light generated by Firefly and Renilla luciferases were detected 24 hours after co-transfection of reporter construct and relevant miRNA, mimic or miR-non-targeting control in 3T3-L1 cells. The method can determine the specificity of miRNA interaction site but will not specify where it binds exactly, however if the 3'-UTR sequence is mutated at the miRNA recognition site it can provide information about the binding site. Other ways to validate targets are based on the assumption of the interaction of miRNA and AGO protein forming

miRISC, as described in section 1.6.1.1. Techniques based on RNA immunoprecipitation will isolate miRISC by targeting AGO thereafter RT-qPCR or sequencing can be evaluated for determination of the binding (87–89),

3.4.3 Functional analyses

In all studies the hypothesis of miRNA action was functionally evaluated by modulating expression levels of miRNA *in vitro*. For induction of miRNA expression mimics reagents are used, these mimics naturally appearing miRNAs and are chemically manufactured double stranded oligonucleotides. To reduce the miRNA expression inhibitors products are used and they consist of single stranded RNA. Mimics/inhibitors are loaded to RISCs which are responsible for the action. The negative control are similar molecules but with no target. The main drawback with miRNA mimics or inhibitors are the off-target-effects. Therefore one needs to control the transfection by checking the miRNA expression, possible measure already known targets and preferable control that the miRNA binds to the gene of interest. Also, depending on the natural expression of the miRNA, when miRNA is highly expressed it's more convenient to inhibit the expression and *vice versa*, if the miRNA is exhibiting low expression it is more rational to overexpress it. In the studies I-II results on both mimics and inhibition reagents are reported, e.g. miR-92a-3p (study I) and miR-193b-3p (study II).

The pipeline after treating cells (independently of treatment) the samples were collected for RNA isolation, cDNA synthesis and assessment of mRNA expression using RT-cPCR to ensure effects of the overexpression, inhibition or gene silencing. The next step is to investigate if changes in mRNA levels can be detected on protein levels and this step is usually more problematic. Although mRNA levels will give an indication of protein changes, studies show that the correlation are with RT-qPCR and protein levels are quiet poor (90). Therefore it is highly recommended to measure both mRNA and proteins if possible. The reason for low correspondence between mRNA and protein is mainly due to the complex features of proteins. Proteins vary in half time; post-translational modification, conformational folding and specific time-points can be crucial for detection of protein expression.

3.4.3.1 Expression determination

In this thesis, levels of miRNAs/mRNAs were determined using conventional methods such as RT-qPCR. For the expression quantification we amplified mRNA using Taqman and Sybro green assay. The principles of Taqman assays are that a specific probe is labelled with a fluorescent dye and quencher. The probe binds specifically to cDNA, which will be cleaved once the polymerase reaches the probe. Consequently, the releasing the flourophore from the probe and fluorescence will be emitted. The manufactures of the probes more are expensive compared to SYBR green assay. In this method the fluorescent DNA binding dye is non-specific and binds to all double-stranded DNA during elongation. Thus, there is a high rate of false-positive PCR-products and creation of primer-dimers. SYBR green

primers need to be optimized before use by careful primer design and use of melting curves and gel. In both technologies a PCR apparatus detects fluorescense. In all experiments a reference gene was amplified in parallel, either 18S RNA or LRP10.

3.4.3.2 Protein assessment

For assessment of protein two conventional methods were used, Western blot and enzyme-linked immunosorbent assay (ELISA). In both methods, a highly specific antibody is required. Western blot is considered to be a semi-quantitative method and the data provided gives a relative comparison of the protein levels and no measure of quantity is assessed. Western blot is an old and quite robust method, however it involves many steps and is time consuming and successful outcome is directly depend on the specificity of antibodies.

3.4.3.2.1 **ELISA**

ELISA is a quantitative method for the determination of the amount of any particular protein in a mixture. All the ELISAs used in the presented studies are commercially available and based on a sandwich enzyme immune assay. ELISA requires a low amount of sample and is simple to perform. The drawback of ELISA is highly dependent on antibody-protein interaction, so if the binding would be unspecific this will not be verified. To avoid this, competitor ELISA can be used to detect the specificity of the antibody. The principle of competitor ELISA is that the sample antigen is incubated with the primary antibody for interaction. The complex is then added to wells coated with the same antigen. If the initial concentration of the antigen is high, there will be a smaller quantity to bind the primary antibody on the well, resulting in a reduced signal.

In paper I-II, ELISA was used to assess amounts of secreted CCL2 and adiponectin proteins into conditional medium. In study IV, ELISA was also used but here we used cell lysates for evaluate post-translational phosphorylation of the proteins involved in insulin signaling.

3.4.3.2.2 **Western blot**

For western blot we used whole or less complex nuclear lysate, described in the papers. The western blot includes three major steps. First the cells are lyzed in lysis buffer containing detergents and to solubilize the proteins. For nuclei preparation from adipocytes, cells were prepared based on swelling in hypotonic buffer, allowing disruption of cellular membrane but leaving nuclei intact. Nuclei are separated from cytosolic fraction by sedimentation followed by lysis generating soluble fraction of nuclear proteins. The proteins are separated by SDS-PAGE by size and transferred to a membrane followed by Western blot procedures. Specifically, the unspecific sites on membrane are blocked using various blocking agents such as BSA or non-fat milk followed by incubation with primary and secondary antibodies of interest. The antigen-antibody complexes are detected using chemiluminiscence, the intensity of the bands are evaluated by densitometry and the relative levels of proteins are calculated in relation to loading control proteins such as actin of lamin A/C.

3.4.3.3 Insulin signaling

In manuscript IV, we studied insulin sensitivity in fat cells and evaluated ability of cells to incorporate triated glucose into lipids. Total glucose uptake into lipids was also performed in *in vitro* differentiated adipocytes with modulated levels of miRNA. The rationale behind the use of glucose is that it is incorporated into lipid and rate limiting for lipogenesis. This is a well-established method and for a more detailed description see (91,92).

4 RESULTS AND DISCUSSION

In this section, the most remarkable findings are summarized and discussed, a complete description of the results is found in each respective paper. Studies I-III are follow-up projects from our lab, with the same patient cohort, but with novel individual hypothesis. The study by Arner et al. was among the first to investigate obesity-dysregulated miRNAs and their link to inflammation (73). In there, gene and miRNA expression were profiled in scWAT obtained from 56 subjects (cohort 1). Adipocyte-specific transcription regulatory network (TRN) controlling *CCL2* expression in obesity was constructed based on the altered expression of miRNAs and altered activity of TFs in obese vs. non-obese, later determined by Motif activity response analysis (MARA) (73). Briefly, MARA is a method developed to find key regulatory elements that identify active promoters and align them with defined motifs followed by calculation of motif activity (93). Therein, 11 miRNAs were discovered to be significantly dysregulated in obesity, affecting CCL2 production directly or indirectly through TFs (73).

In study I, the TRN regulating CCL2 was extended and validated. Interacting circuits of miRNAs and TFs were further verified in human adipocytes and macrophages. In study II, we investigated if the obesity-dysregulated miRNAs affected adiponectin production in human adipocytes. For study III, we aimed to discover miRNAs associated with differences in WAT morphology. In the fourth study we intended to identify miRNAs involved in the development of fat cell insulin sensitivity. An overview of the presented studies in this thesis is illustrated in figure 3.

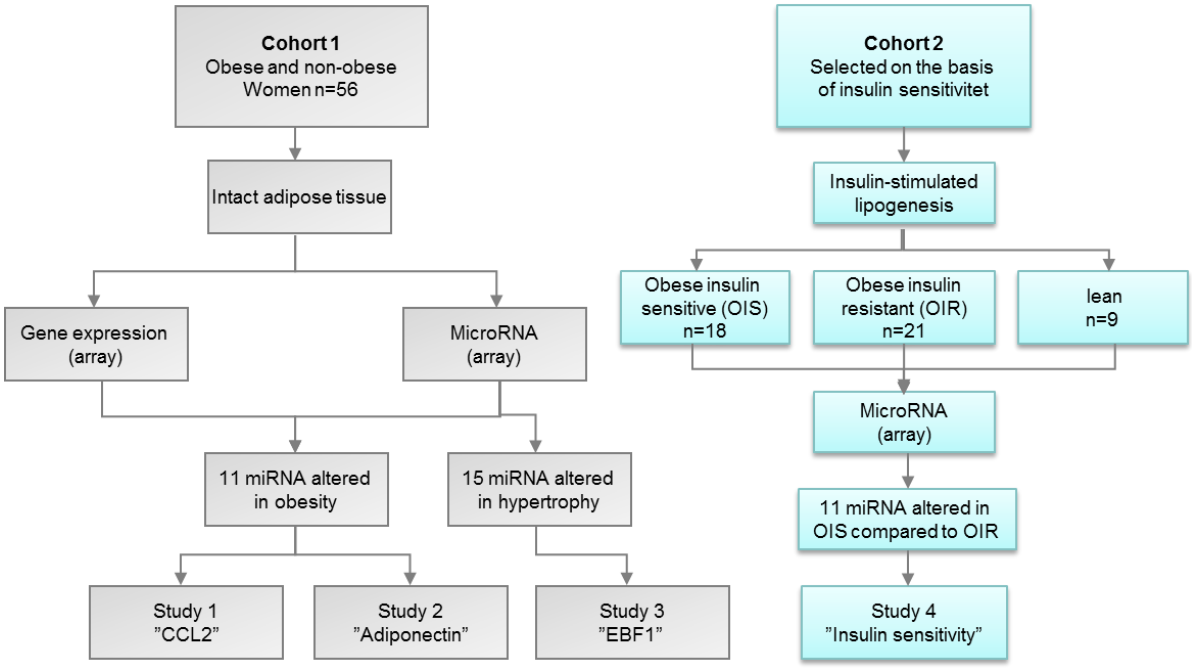

Figure 3. An outline of the study strategies presented in this thesis**.**

4.1 STUDY I

As mentioned above, study I is based on previous investigations from our lab, defining miRNAs possible role in controlling CCL2 production (73). By using a systematic unbiased approach, 10 miRNAs were demonstrated to affect CCL2 production, and for two of them (miR-126-3p and -193b-3p) the direct or indirect regulatory circuits were defined. For the first study, we extended and validated the above described miRNA network. We used gain-off-function/loss-of-function techniques to examine the impact of TFs and miRNAs on CCL2 expression in human adipocyte and macrophages. We focused on a part of the TRN investigating the regulatory pathway of miR-92a-3p which was not fully characterized in the previous study. Firstly, we investigated if miR-92a-3p affected its predicted target, specificity protein 1 (SP1) and v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (*ETS1*). Alteration of miR-92a expression affected mRNA and protein expression of SP1 but not ETS1 and miR-92a-3p interacted directly with the 3'-UTR region of SP1. SP1 alone regulated CCL2 production as evidenced by decreased CCL2 production after silencing of SP1 and reduced expression of v-rel reticuloendotheliosis viral oncogene homolog (avian) (*REL*), which is a part of the nuclear factor (NF)-κB family.

In the previous study, miR-193b-3p was shown to regulate CCL2 through ETS1 and MYC-associated factor X (MAX) (73). We wanted to evaluate if these TFs could affect CCL2 without the impact of miR-193b-3p. ETS1 knock down decreased mRNA levels of *MAX*, signal transducer and activator of transcription 6, interleukin-4 induced, nuclear factor of k light polypeptide gene enhancer in B-cells 1, and *CCL2*. Silencing the expression of MAX diminished the mRNA levels of *RELB*. The findings lead us to hypothesize that the TFs might have synergistic effects on the CCL2 production, which were confirmed by co-knock down of ETS1 and MAX.

These observations indicate that miRNAs have combinatorial properties by amplifying the effects of each other (94). Therefore, we tested the effects of co-overexpressing the miRNAs involved in the studied TRN (miR-193b-3p, -126-3p and -92a-3p) in both adipocytes and macrophages. We found that two combinations of miRNAs (miR-193b-3p + 92a-3p and miR-126-3p-3p + 92a-3p) reduced CCL2 expression, while no effect was observed for miR-126-3p + 193b-3p in adipocytes. On the contrary, only a combination of miR-126-3p + 193b-3p had effect on CCL2 production in macrophages.

Taken together, the novelty of this study is that the regulatory interactions between miRNAs, and TFs were determined in different cell types e.g. adipocytes and macrophages, shown in Figure 4. Understanding the miRNAs individual function as well as the interaction with other miRNAs, exhibiting synergistic effects, is key in order to get an overview of the regulation. We studied two levels of regulation including TFs and miRNAs, however the TRN could be extended, including more levels of regulation such as methylation and phosphorylation. A more comprehensive investigation to determine the mechanistic crosstalk between macrophages and adipocytes is one more possibility to extend the study. Here we examined

only scWAT obtained from female donors and further studies can be addressed to find possible sex- and region- specific differences in the regulation of CCL2 production.

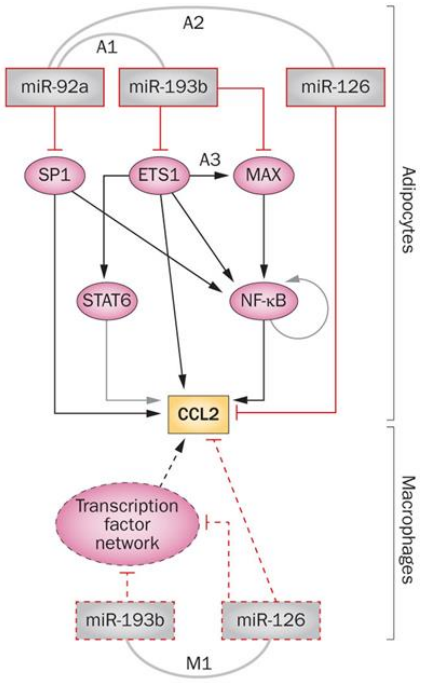

Figure 4. miRNA regulatory circuits that control levels of CCL2 in human white adipose tissue. Transcription factors (ETS1, MAX, SP1 and an NF-κB subnetwork) and miRNAs (miR-92a-3p, miR-126-3p and miR-193b-3p) are integrated in a regulatory network of CCL2. The experimentally verified network demonstrates that miRNAs regulate CCL2 in additive manner. In adipocytes, two combinations was identified; miR-92a-3p + miR-193b-3p acts through a transcriptional regulatory network to control CCL2 (A1); miR-126-3p acts directly on CCL2 but also together with miR-92a-3p through transcription factors (A2). The transcription factors ETS1 and MAX demonstrate synergistic effects in their control of CCL2 (A3). In macrophages, miRNAs could act directly or indirectly regulating CCL2. T bars indicate inhibition; arrows indicate stimulation. Bold lines represent interactions between network players in adipocytes and thin lines are interactions predicted by network analyses and/or shown in other cell types. Dashed lines in the lower part represent possible interactions. The networks presented do not include transcription factors without known DNA-binding motifs and co-regulators of transcription factors. Source: Nat Rev Endocrinol. 2015;11:276-88 (57). Printed with minor changes and with permission from the publisher.

4.2 STUDY II

The beneficial metabolic effects of adiponectin have been intensively investigated although the molecular mechanisms regulating the expression of this adipokine are not well mapped. In specific, there are not much data available on adiponectin regulation by miRNAs. Therefore the objective of study II was to determine whether adipocyte-expressed miRNAs altered by obesity can regulate adiponectin expression/secretion in fat cells. Eleven miRNAs previously shown to be dysregulated in obese human WAT were overexpressed in human *in vitro* differentiated adipocytes followed by assessments of adiponectin levels in conditioned media.

Three miRNAs (miR-193b-3p/ -126-3p/ -26a-5p) increased the adiponectin levels in conditioned medium. However, only miR-193b-3p demonstrated clinical relevance as its

expression correlated with adiponectin gene expression and measures of insulin resistance ($HOMA_{IR}$) in the clinical cohort of 56 subjects. In general, miRNAs down-regulate their predicted targets. As we found that miR-193b-3p overexpression up-regulated adiponectin secretion indicated that the mechanisms linking the miRNA to adiponectin were indirect. Moreover, the effects are probably via upstream negative regulators (e.g. miRNA→intermediate regulator→*ADIPOQ)*. Our hypothesis was supported by bioinformatics analyses, in which *ADIPOQ* was not a direct target of miR-193b-3p. This prompted us to search for predicted miR-193b-3p targets among genes known to affect adiponectin production. We identified 11 negative regulators of adiponectin that were also predicted miRNA-193b-3p targets. Even though the adiponectin-regulating genes were predicted targets of miR-193b-3p, they were not validated experimentally. Therefore, we measured the mRNA expression of the genes (adiponectin regulators) in adipocytes with altered levels of miR-193b-3p. Overexpression of miR-193b-3p decreased the abundance of cAMP-responsive element binding protein 5 (*CREB5*), nuclear transcription factor Y α (*NF-YA*), nuclear receptor interacting protein 1 (*NRIP1*), protein kinase D3 and retinoid X receptor-α, suggesting that the genes are potential targets of miR-193b-3p. However, 3'-UTR reporter analysis confirmed that only CREB5, NF-YA and NRIP1 were direct targets of miR-193b-3p.

The effect of miR-193b-3p on NF-YA was confirmed at the protein level whereas CREB5 protein, in contrast to the findings at the mRNA level, was increased. All tested primary antibodies detecting NRIP1 were unspecific in human adipocytes and therefore data cannot be demonstrated. To strengthen the mechanistic part of evidence, we studied the interaction of miR-193b-3p with NF-YA and NRIP1 by co-transfecting *in vitro* differentiated adipocytes with miRNA mimics and target protectors corresponding to the binding site of miR-193b-3p on the 3'-UTR of NF-YA or NRIP1 followed by the evaluation of adiponectin secretion. Target protectors are small RNA molecules specific for a single target gene and miRNA. They specifically interfere with the interaction between a miRNA and its target sequence. Indeed, co-transfection of the target protectors eliminated the effect of miR-193b-3p on adiponectin production

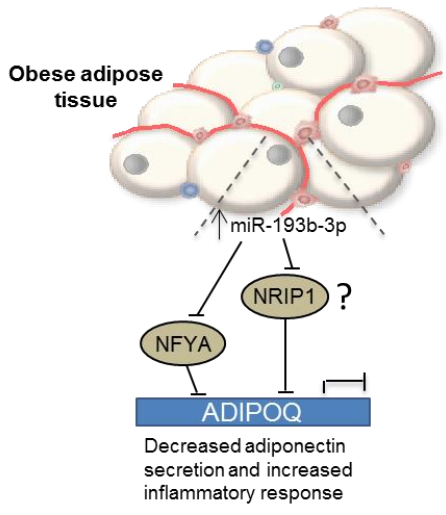

Figure 5. The suggested regulatory role of adiponectin by miRNA in human fat cells**.** The pathway of miR-193b-3p towards adiponectin regulation was experimentally verified, indicating that NF-YA is and possibly NRIP1 are the intermediate players directly targeted by miR-193b-3p.

Research in the adiponectin field includes the regulation of miRNAs by adiponectin or the regulation of adiponectin receptor expression by miRNAs (95–99). Our findings contribute with a novel regulatory layer, specifically the regulation of adiponectin by miRNA in human adipocytes and are illustrated in figure 5. Altogether, the results recognize a clinically relevant miR-193b-3p that is downregulated in obesity and controls adiponectin expression through binding of NF-YA and probably NRIP1.

4.3 STUDY III (MANUSCRIPT)

WAT morphology characterized by hypertrophy associates with increased adipose inflammation, lipolysis, insulin resistance, and risk of diabetes. However, the causal relationships and mechanisms controlling WAT morphology are unclear. Our group recently identified EBF1 as an adipocyte expressed TF with decreased expression/activity in WAT hypertrophy (42). In human adipocytes, the regulatory targets of EBF1 were enriched for genes controlling lipolysis and adipocyte morphology/differentiation and reduced EBF1 levels were associated with increased lipolysis and adipose hypertrophy. However, the mechanisms controlling EBF1 expression are not known, neither any miRNAs linked to human WAT morphology have been identified. Therefore, we aimed to identify miRNAs associated with differences in WAT morphology and determined whether they could affect the expression of EBF1.

With this aim we analyzed previously published miRNA expression data from 26 non-obese individuals subdivided into hypertrophy (n=13) or hyperplasia (n=13) (42,100). We focused on the miRNA expression in lean individuals due to the fact that fat cell morphology is more evident in lean than obese. The analysis allowed us to identify 15 differentially regulated miRNAs (miR-574-5p, -361-5p, -143-3p, -222-3p, -221-3p, -125b-5p, -106a-5p, -26a-3p, -23a-3p, -16-5p, -17-5p, let-7a, -7c-5p, -7d and -7i-5p), all of which were significantly highly expressed in hypertrophy. Interestingly, ten miRNAs (let-7a, -7c-5p, -7d, -7i-5p and miR-106-5p, -143-3p, -221-3p, -23a-3p, -361-5p and -574-5p) were predicted to target EBF1. This finding prompted us to perform 3'-UTR analysis of EBF1 and above mentioned miRNAs. Overexpression of miR-361-5p and -574-5p caused a significant downregulation of luciferase reporter activity, indicating that EBF1 is a true target for these miRNAs in adipocytes. For functional studies of the miR-361-5p and -574-5p, we overexpressed each miRNAs independently which consequently reduced *EBF1* levels by 15-24 %. We also tested if the miRNAs (miR-361-5p and 574-5p) could possess additive effects on EBF1 regulation. Indeed, co-transfection of both miR-361-5p and -574-5p using low concentrations of mimic reagents resulted in a more pronounced reduction of EBF1 expression (~15 %) compared with individual overexpression of either miRNAs.

We tested if overexpression of miRNAs reduced proteins levels of EBF1. However, at this point, we could not detect the same amount of EBF1 protein reduction as was detected on

mRNA levels. The lack of difference in EBF1 protein levels could depend on low affinity and/or specificity of primary antibodies used. Another explanation could be long half-life of EBF1 protein. Previous study by Rosen et al. demonstrated that EBF1 has a half time of seven days (101). Since EBF1 is also an important factor for adipogenesis, it is not reasonable to manipulate levels of EBF1 at early stages of differentiation due to possible anti-adipogenenic effects. To overcome the limitations we are currently investigating other ways to quantify EBF1 protein levels, testing new antibodies and investigating if the protein changes could be more profound in the nuclear lysates instead of total cell lysate.

In the previous study by Gao et al. the factor Cell Death-Inducing DFFA-Like Effector A (CIDEA) was regulated by EBF1 (42). CIDEA is known to be exclusively expressed in human WAT and an important metabolic factor, controlling lipolysis and TNF-α secretion (102). We therefore hypothesized that CIDEA could possibly be altered as a secondary effect of the studied miRNAs. Indeed, *CIDEA* mRNA levels were reduced by overexpression of miR-361-5p and -574-5p. Because EBF1 is a transcription factor and its expression is altered in obesity, its activity was determined by MARA in the previous study (73). We used the data to perform an association analysis that revealed significant and negative correlation with the expression of both miRNAs in fat cells independently of BMI.

In summary, we identified miR-365-5p and -574-5p that could be linked to WAT hypertrophy and their potential role in regulating fat cell morphology due to their attenuating effects on EBF1 expression (Figure 6). These miRNAs could therefore have a pathophysiological relevance and play an important role for the development of insulin resistance. An overview of the obesity dysregulated miRNAs in WAT recently obtained by co-workers of our group is summarized in table 3.

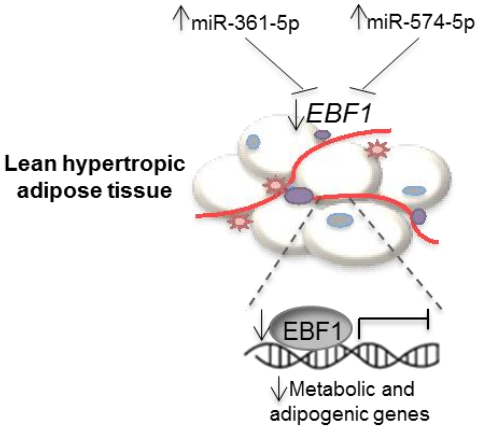

Figure 6. Proposed pathway of EBF1 regulation by miR-361-5p and -574-5p in hypertrophic human WAT. Both miR-361-5p and -574-5p bind and thereby regulate EBF1 independently and in an additive manner.

4.4 STUDY IV (MANUSCRIPT)

In this study we aimed to gain insight into the regulation of fat cell insulin sensitivity by miRNAs. So far there is only a handful of studies of miRNAs in relation to IR of human fat cell (103,104). We selected obese individuals based on insulin sensitivity that was determined

by insulin-stimulated minus basal lipogenesis of isolated fat cells. This way we could generate the cohort consisting of 21 OIS, 18 OIR and 9 lean subjects as controls. The obese groups were matched for BMI, waist circumference and fat cell size, all of which may influence insulin sensitivity independently of lipogenesis values. More clinical data could be found below in the manuscript of study IV.

A combination of global miRNA measurements and validations of miRNA expression with RT-qPCR identified eleven adipose miRNAs to be differently regulated in IOS compared to IOR. These candidate miRNAs were further pursued for functional studies *in vitro*. The miRNAs were overexpressed in human differentiated adipocytes followed by measurements of basal and insulin-stimulated glucose incorporation of lipids. Two miRNAs (miR-143-3p and miR-652-3p) increased glucose incorporation into lipids in response to insulin.

The possible clinical impact was evaluated by performing association analysis of the miRNAs expression determined by RT-qPCR in intact WAT with the lipogenesis values in isolated fat cells *ex vivo*. Indeed, expression of both miR-143-3p and miR-652-3p correlated to insulin-stimulated lipogenesis independently of BMI. To identify targets of miR-143-3p and miR-652-3p involved in lipogenic pathways and to delineate their possible regulatory pathways, we selected for known genes involved in insulin signaling, insulin sensitivity/resistance and lipogenesis. To identify if these genes could possibly be targeted by the miRNAs, they were overlapped with the list of predicted targets of the miR-143-3p and miR-652-3p. We identified 13 and 8 genes for miR-143-3p and miR-652-3p respectively thus, mRNA expression was measured after overexpression of the miRNAs in adipocytes. Overexpression of miR-143-3p increased PRKAA1 mRNA levels by about 35 % while overexpression of miR-652-3p downregulated ENPP1 by 30 %. The findings were also confirmed at the protein levels for both genes. As miR-652-3p reduced its predicted target, one could suspect that it directly targeted ENPP1. Indeed, the direct binding of miRNA- 652-3p to the 3'-UTR of ENPP1 was confirmed by the luciferase reporter assay.

As mentioned in section 1.3.1.1, insulin signaling comprises of numerous phosphorylation events and the protein kinase AMPK is an important regulator for energy metabolism responsible for direct phosphorylation of metabolic enzymes. PRKAA2 is a catalytic subunit of AMPK. The fact that overexpression of miRNA-143-3p increased expression of PRKAA2 allowed us to hypothesize for indirect effects where posttranslational events could be involved. Obviously, miRNA-652-3p also targets multiple targets, many of them indirectly. Thus we overexpressed both miRNAs in adipocytes, and measured total and phospho-proteins at the activating residues of the main insulin signaling players using ELISA (Akt2, phospho-Akt2, total IRS-1, phospho-IRS1, Insulin Receptor β and phospho-Insulin Receptor β). We found that phosphorylation of AKT2 at Ser474-site was induced by miR-143-3p and miR-652-3p increased phosphorylation of AMPKα at activating residue Thr172 and total phosphorylation of IRS-1.

In summary, miR-143-3p and miR-652-3p were downregulated in OIR compared to OIS

women. We found that they increase insulin-stimulated lipogenesis directly by reducing ENPP1 and indirectly by inducing post-translational (PRKAA2) and phosphorylation's events. The main findings are illustrated in a schematic picture (Figure 7).

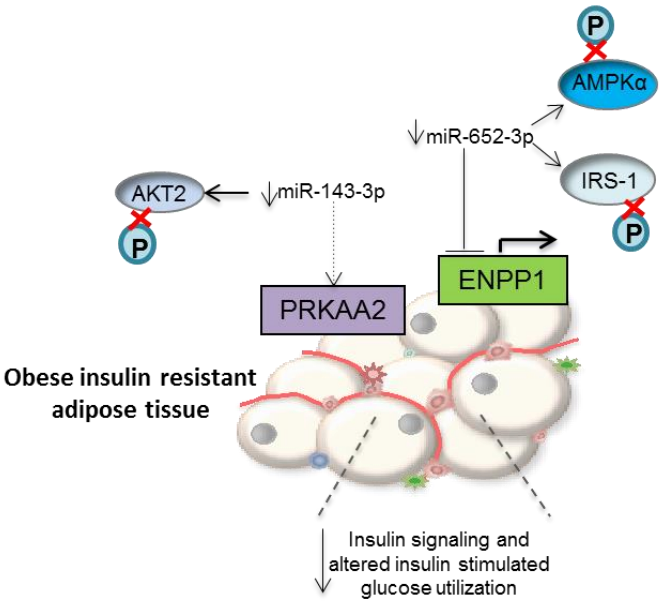

Figure 7. A schematic summarization of miRNAs involved in glucose incorporation in human white adipocytes. Two miRNAs was found to be downregulated in obese insulin resistant adipose tissue and suggested to regulate lipid incorporation. The circuit of the experimentally validated miRNAs indicated that miR-143-3p indirectly affects PRKAA2 (indicated by dashed line), whereas miR-652-3p was found to bind and regulate ENPP1. Both miRNAs was moreover engaged in phosphorylating important insulin signaling factors such as AKT2, AMPKα and IRS-1 through undefined mechanism.

Here, we included phosphorylation events that positively regulate insulin signaling due to the stimulatory effects of the miRNAs on glucose incorporation to the lipids. Phosphorylation of residues promoting inhibitory effects is also important but was not covered in here. It would be interesting to more thoroughly investigate and characterize miRNAs effect on insulin signaling post-translationally and especially the phosphorylation actions.

Table 3. An overview of the obesity dysregulated miRNAs in WAT and their effect on adipocyte function.

MicroRNA	Expression in WAT			Combinatorial effect	Affect			Effect on Lipolysis
	Obese/lean	IS/IR	Morphology		CCL2	Adiponectin	TNF- α	
Let-7a-5p	down	-	-	-	yes	no	Yes	no
Let-7d-5p	down	-	up in hypertrophy	-	yes	no	yes	yes
miR-26a-5p	down	down	up in hypertrophy	-	yes	yes	yes	yes
miR -30c-5p	down	-	-	-	no	no	no	yes
miR -92a-3p	down	-	-	on CCL2 (miR-92a+193b/126)	yes	no	yes	no
miR -126-3p	down	-	-	on CCL2 (miR-126+92a)	yes	yes	no	no
miR -143-3p	down	down	up in hypertrophy	-	yes	no	yes	no
miR-145-5p	down	down	-	-	yes	no	yes	yes
miR-193a-5p	down	-	-	-	yes	no	yes	no
miR-193b-3p	down	-	-	on CCL2 (miR-92a+193b)	yes	yes	yes	yes
miR-652-3p	down	down	-	-	yes	no	no	yes
miR-361-5p	-	-	up in hypertrophy	on EBF1 (miR-361+574)	-	-	-	-
miR-574-5p	-	-	up in hypertrophy	on EBF1 (miR-361+574)	-	-	-	-
References	Arner *et al.* (2012) (73)	Dahlman et al. (MS)	Belarbi *et al.* (Manuscript 2016)	Kulyté *et al.* (2014) (105), Belarbi *et al.* (Manuscript 2016)	Kulyté *et al.* (2014) (105)	Belarbi *et al.* (2015)	Lorente-Cebrian *et al.* (2014) (106)	Lorente-Cebrian *et al.* (2014) (106)

Abbreviations: IS, insulin sensitive; IR, insulin resistance; CCL2, chemokine (C-C Motif) Ligand 2; TNF- α, tumor necrosis factor α; EBF1, early B cell factor 1.

5 CONCLUSION AND FUTURE PERSPECTIVES

WAT has a remarkable ability to adapt and remodel in response to under- and over nutrition. The flexibility of WAT includes alterations in the structure and composition that consequently affects adipocyte metabolism. The biology of the different conditions of WAT is complex and extensive research is performed in order to understand the molecular events and its clinical impact. The focus of this thesis was to identify miRNAs in WAT and map their regulatory pathways in relation to obesity and IR. Our results provide novel insights on the function of miRNAs in human adipocytes. Nevertheless, many questions remain to be addressed.

Future investigations are needed to evaluate the clinical impact of the dysregulated miRNAs and to elucidate the complete gene regulatory networks in WAT and their contribution to insulin sensitivity and inflammation. For all four studies, the experimental pipeline was to identify miRNAs by global expression analysis of the miRNA transcriptome. Further finding and validating comprehensive pathways leading to clinical pathologies is even more important. **Studies I-III** are based on the same cohort (cohort 1) consisting of lean and obese individuals, where expression analysis of miRNA and genes were assessed. Data revealed that 11 miRNAs were dysregulated in obesity (73). We further studied miRNAs and miRNA-gene regulatory networks to get insights into different aspects of WAT function, e.g. inflammation (studies on CCL2 and adiponectin) and WAT morphology (EBF1 study). **In study IV**, we evaluated the impact of miRNAs on insulin sensitivity in obese subjects. Altogether, these studies demonstrate the impact of miRNAs in regulating WAT function.

A further apparent and very relevant investigation is to create networks that include additional levels of regulation including chromatin marks (DNA methylation, histone modifications) and post-translational modifications such as phosphorylation and methylations events. In addition, it would be of interest to map the obesity-regulated miRNAs which were excluded from more detailed analyses due to various reasons. By including these miRNAs, the regulatory network could be extended. For example in study III it would be interesting to study the remaining 9 miRNAs, and how they may be involved in regulating WAT morphology through e.g. *EBF1*, *PPARG*, and C/*EBPα*. These three genes encode TFs that cross talk and regulate each other and all are downregulated in hypertrophic non-obese WAT (42,101). In general, studies on the regulation of miRNAs themselves are lacking and it would be highly relevant to consider this extra layer. Other aspects of interest are the ability of miRNAs to act in a combinatorial manner and to characterize if/how miRNAs crosstalk with other tissues e.g. through exosome secretion.

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